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(54) Method for producing L-threonine by fermentation.

(57) New L-threonine-producing strains of the genus Brevibacterium and Corynebacterium are obtained by separating a plasmid DNA from a microorganism of the genus Brevibacterium and Corynebacterium, inserting into the plasmid DNA a fragment of chromosomal DNA derived from a DNA-donor strain of the genus Brevibacterium or Corynebacterium resistant to α-amino-β-hydroxy valeric acid to obtain a recombinant plasmid DNA, incorporating the recombinant plasmid DNA into a recipient strain of the genus Brevibacterium or Corynebacterium which is sensitive to α-amino-β-hydroxy valeric acid and isolating a strain transformed to become resistant to α-amino-β-hydroxy-valeric acid.

By aerobically cultivating the new strains in an aqueous culture medium L-threonine may be obtained in high yields.

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TITLE OF THE INVENTION:

METHOD FOR PRODUCING L-THREONINE BY FERMENTATION

BACKGROUND OF THE INVENTION

Filed of the Invention:

The present invention relates to a method for producing L-threonine by fermentation, and particularly relates to a method for producing L-threonine with a microorganism of the genus Brevibacterium and Corynebacterium constructed by a gene recombination technique.

Description of the Prior Art:

Hitherto, in order to render a wild strain capable of producing L-threonine from carbohydrates, it has been necessary to induce artificial mutants from the wild strain. There are many known L-threonine-producing artificial mutants. Most of the known threonine-producing mutants are resistant to α-amino-β-hydroxy valeric acid (hereinafter referred to as "AHV"), and belong to the genus Brevibacterium or Corynebacterium. These microorganisms produce L-threonine in a yield of from 10 to 20%.

Examples of publications are U.S. Patent No. 3,582,471, U.S. Patent No. 3,580,810 and Japanese Publication No. 47-34956, in which threonine producing mutants resistant to AHV and belonging to the genera Brevibacterium, Escherichia and Corynebacterium are disclosed, respectively. Recent publications concerning the threonine production especially by mutants of the genera Brevibacterium and Corynebacterium are Japanese Published Unexamined Patent Applications Number 51-54984, 53-101591, 54-32693, 54-35285, 54-35286, 54-35288, 54-37886 and 54-92692.

Another approach to increase the productivity of threonine in microorganisms is found in U.S. Patent No. 4278765 and Japanese Published Unexamined Patent Application Nos. 55-131397 and 56-15696, in which threonine producing Escherichia coli strains transformed with a recombinant plasmid DNA and then constructed by a gene-recombination technique were disclosed.

However, it has been difficult to construct commercially applicable threonine-producers of Escherichia coli by the gene-recombination technique, because originally Escherichia strains can not express high productivity of L-threonine and the recombinant strains derived from such Escherichia strains can not produce high amounts of L-threonine.

On the other hand, there are many strains within the genera Brevibacterium and Corynebacterium which produce high amounts of L-threonine, and therefore strains of Corynebacterium and

Brevibacterium may be suitable as the original strain for the construction of L-threonine-producers by a gene-recombination technique. However, although the presence of plasmids in the strains of Brevibacterium and Corynebacterium is known (Publication of European Patent Application No. 0030391), the plasmids have no specific characteristics to be used as the marker for identification of the plasmids, and therefore it has been very difficult to select recombinant plasmids, derived from the plasmids of Brevibacterium and Corynebacterium. For the above reason it has been difficult to construct L-threonine-producers from the L-threonine producing strains of Brevibacterium and Corynebacterium by the gene-recombination technique.

Therefore there still exists a need for the development of a novel process for the production of L-threonine in high yields.

SUMMARY OF THE INVENTION

It is therefore an object of the present invention to provide a novel method for producing L-threonine by fermentation in high yields.

The inventors have found that strains of the genus Brevibacterium or Corynebacterium transformed to L-threonine-producers can be isolated by selecting strains transformed to become resistant to AHV.

There is now provided a method for producing L-threonine by fermentation which comprises aerobically culturing in an aqueous

culture medium an L-threonine producing microorganism obtained by isolating a strain transformed to become resistant to AHV after incorporation into a recipient strain of the genus Brevibacterium or Corynebacterium which is sensitive to AHV, of a plasmid DNA obtained from a microorganism of the genus Brevibacterium or Corynebacterium and having been inserted therein a fragment of chromosomal DNA derived from a DNA-donor strain of the genus Brevibacterium or Corynebacterium which is resistant to AHV, and recovering L-threonine accumulated in the resulted culture liquid.

The present invention also provides a method for constructing an L-threonine producing strain which comprises:

(a) separating a plasmid DNA from a microorganism of the genus Brevibacterium and Corynebacterium,

(b) inserting into the plasmid DNA a fragment of chromosomal DNA derived from a DNA-donor strain of the genus Brevibacterium and Corynebacterium resistant to AHV to obtain a recombinant plasmid DNA

(c) incorporating the recombinant plasmid DAN into a recipient strain of the genus Brevibacterium or Corynebacterium which is sensitive to AHV, and

(d) isolating a strain transformed to become resistant to AHV.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The DNA-donor strain used to construct the L-threonine producer of this invention is a mutant of the genus Brevibacterium or Corynebacterium resistant to AHV. Strains having higher productivity of L-threonine are used preferably as the DNA-donor. The mutant resistant to AHV used as the DNA-donor can be obtained by conventional mutation techniques such as exposing the parent strain to 250μg/ml of N-methyl-N'-nitro-N--nitrosoguanidine in a buffer solution and separating the colony which appeared on the agar medium containing an amount of AHV inhibitive to the growth of the parent strain. Such DNA-donor naturally has a chromosomal DNA region controlling the AHV resistance.

Known strains resistant to AHV are, for example, mutants of Brevibacterium resistant to AHV (Japanese Published Examined Patent Application 26708/1970,mutants of Corynebacterium resistant to AHV (Japanese Published Examined Patent Application 34956/1972),mutants of Brevibacterium resistant to AHV and requiring L-lysine, L-methonine or L-isoleucine (Japanese Published Examined Patent Application 44876/1973), mutants of Brevibacterium resistant to AHV and decoyinine or psicofuranine (Japanese Published Unexamined Patent Application 32693/1979), mutants of Brevibacterium resistant to AHV and α-chlorocaprolactam, γ-methyllysine or γ-carbobenzoxylysine (Japanese Published Unexamined Patent Application 35285/1979),mutants of

Brevibacterium resistant to AHV and requiring alanine (Japanese Published Unexamined Patent Application 35286/1979), mutants of Brevibacterium resistant to AHV and S-methylcysteinesulfoxide, S-methylglycinesulfoxide, carbobenzoxyvaline, carbobenzoxythreonine or N-benzoylallothreonine (Japanese Published Unexamined Patent Application 35288/1979), mutants of Brevibacterium resistant to AHV and p-chloro-m-fluoromethyl phenylalanine (Japanese Published Unexamined Patent Application 37886/1979) and mutants of Brevibacterium resistant to AHV and leucine-analogue (Japanese Published Unexamined Patent Application 92692/1979).

Other than the DNA-donors listed above, AHV-resistant strains can be obtained by introducing AHV-resistance by a conventional manner into a so-called Coryne-form glutamic acid producing bacteria, of which typical strains are shown below:

Brevibacterium divaricatum ATCC 14020

Brevibacterium saccharoliticum ATCC 14066

Brevibacterium immariophilum ATCC 14068

Brevibacterium lactofermentum ATCC 13869

Brevibacterium roseum ATCC 13825

Brevibacterium flavum ATCC 13826

Brevibacterium thiogenitalis ATCC 19240

Corynebacterium acetoacidophilum ATCC 13870

Corynebacterium acetoglutamicum ATCC 15806

Corynebacterium callunae ATCC 15991

Corynebacterium glutamicum ATCC 13032

Corynebacterium lilium ATCC 15990

Corynebacterium melassecola ATCC 17965

As the vector DNA, plasmids obtained from the Coryne-form glutamic acid producing bacteria of the genera Brevibacterium and Corynebacterium or their mutants, and derivatives of the plasmids can be used. Specimens of the plasmids are pAM 286 and pAM 330, and pHM 1519.

DNA-recipient used in the present invention are strains sensitive to AHV and belonging to the Coryne-form glutamic acid producing bacteria of the genera Brevibacterium and Corynebacterium. Especially, when a mutant sensitive to AHV which requires L-threonine is used as the DNA-recipient, it is favourable to distinguish the threonine-producing transformant from the recipient, although the threonine-producing transformant can be distinguished from the recipient by AHV-resistance.

Chromosomal DNA is extracted from the DNA donor in a well known manner and treated with a restriction endonuclease by a well known method (Biochem. Biophys. Acta 383: 457 (1975)).

The vector DNA is also treated with a restriction endonuclease in an analogous manner. Various kinds of restriction endonucleases can be used, if the digestion of the chromosomal DNA is done partially. Thereafter, the digested chromosomal DNA and vector DNA are subjected to to a ligation reaction.

Recombination of DNA to prepare the recombinant plasmid can be carried out by the ligation reaction with a ligase,or by incorporating with terminal transferase deoxyadenylic acid and thymidylic acid, or deoxyguanylic acid and deoxycytidylic acid into the chromosomal DNA fragment and cleaved vector DNA and by subjecting the modified chromosomal DNA fragment and cleaved DNA to an annealing reaction.

The recombinant DNA thus obtained can be incorporated into the DNA-recipient by treating the cell of the DNA-recipient with calcium chloride to increase the permeability as is reported regarding E. coli K-12 (Mandel, M. and Higa, A., J. Mol. Biol., 53, 159 (1970)), or by applying for the incorporation cells of the DNA-recipient at a specific stage of growth when cells become capable of incorporating plasmids (competent cell) as is reported with Bacillus subtilis (Duncan, C.H., Wilson, G.A. and Young, F.E., Gene 1, 153 (1977)). The recombinant DNA can also be incorporated into the DNA-recipient by forming protoplast or spheroplast of the DNA-recipient which easily incorporates plasmid DNA as is known with Bacillus subtilis, actinomycetes and yeast (Chang, S and Choen, S.N., Molec, Gen. Genet, 168, 111 (1979)); Bibb, M.J. Ward, J.M. and Hopwood, O.A., Nature, 274, 398 (1978); Hinnen, A., Hicks, J.B. and Fink, G.R., Proc. Natl, Acad. Sci., USA, 75, 1929 (1978)).

The desired transformant can be obtained by isolating the colonies appearing on a medium containing an amount of AHV inhibitive to the growth of the DNA-recipient. Threonine-producers can be obtained from the isolated colonies in high frequency.

The methods of culturing the L-threonine producing strains thus obtained are conventional, and are similar to the methods for the cultivation of known L-threonine producing microorganisms. Thus, the culture medium employed is a conventional one containing carbon sources, nitrogen sources, inorganic ions and, when required, minor organic nutrients such as vitamins or amino acids. Examples of suitable carbon sources include glucose, sucrose, lactose, starch hydrolysate and molasses. Gaseous ammonia, aqueous ammonia and ammonium salts and other nitrogen containing materials can be used as the nitrogen source.

Cultivation of the recombinant microorganisms is conducted under aerobic conditions in which the pH and the temperature of the medium are adjusted to a suitable level and continued until the formation of L-threonine ceases.

The L-threonine accumulated in the culture medium can be recovered by conventional procedures.

By the method of the present invention, L-threonine can be produced in higher yields than has been achieved in previously known methods using artificial mutants of Brevibacterium and Corynebacterium.

Having generally described this invention, a further understanding can be obtained by reference to certain specific examples which are provided herein for purposes of illustration only and are not intended to be limiting unless otherwise specified.

Example 1

(1) Extration of DNA

Corynebacterium glutamicum AJ 11560 (FERM-P 5485) was exposed to 250 µg/ml N-methyl-N'-nitro-N-nitrosoguanidine in 1/10M phosphate buffer of pH 7.2 at 30°C for 30 minutes and the colonies appearing on a minimum medium/(containing, per liter, (M medium) 20g glucose, 10g ammonium sulfate, 2.5g urea, 1g KH_2PO_4, 0.4g $MgSO_4 \cdot 7H_2O$, 50µg biotin, 200µg thiamine·HCl, 0.01g $FeSO_4$, 0.01g $MnSO_4 \cdot 4H_2O$, 1g AHV and 2g agar (pH 7.0)) were isolated as the AHV-resistant strains.

One of the AHV-resistant strains, No. 60 (NRRL B-15046) was cultured at 37°C for 3 hours with shaking in 1ℓ of CMG-medium containing 1g/dl peptone, 1g/dl yeast extract, 0.5g/dl glucose and 0.5g/dl NaCl (pH was adjusted to 7.2), and bacterial cells in the exponential growth phase were harvested. Chromosomal DNA was extracted by a conventional phenol-method, and 0.6mg of purified DNA was obtained.

Corynebacterium glutamicum AJ 11560 was newly isolated as a suitable strain for the purpose of this invenition.

This strain was classified to the section III of the genus Corynebacterium described in Bergey's Manual of Determinative Bacteriology (8th edition, 1974). However, taxonomic characteristics of the species belonging to section III are not disclosed in the Manual, but only disclosed the name of species of section III. Therefore, all original reports disclosed in the Manual as to section III are referred to. AJ 11560 was identified with Corynebacterium glutamicum described in Bull. Agr. Chem. Soc. Japan 22, 176-185 (1958) and J. Gen. Appl. Microbiol., 13, 279-301 (1967).

(2) Preparation of vector DNA

As the vector, DNA of pAM 286, a plasmid of Corynebacterium glutamicum was prepared as follows:

Corynebacterium glutamicum AJ 11560 harboring the plasmid pAM 286 was incubated at 30°C in 1ℓ of CMG medium until the late log phase, cells were harvested and then lysed by treatment with lysozyme and SDS. The lysate was centrifuged at 30,000xg for 30 minutes to obtain the supernatant. After concentrating the supernatant, 60 μg of pAM 286 plasmid DNA was obtained by fractionation using agarose gel electrophoresis.

(3) Insertion of chromosomal DNA fragment into vector.

10 μg of the chromosomal DNA was treated with the restriction endonuclease XbaI at 37°C for 10, 30 and 60 minutes, respectively, to cleave the DNA chains, and then heated at 65°C for 5 minutes, respectively. 5 μg of the vector DNA was

also treated with the restriction endonuclease XbaI at 37°C for 60 minutes to cleave the DNA completely, and then heated at 65°C for 5 minutes.

The digested chromosomal DNA solution and cleaved vector DNA solution were mixed and subjected to the ligation reaction of DNA fragments by a T_4 phage DNA-ligase in the presence of ATP and dithiothreitol at 10°C for 24 hours. The reaction mixture was then heated at 65°C for 5 minutes, and the twofold volume of ethanol was added to it. The precipitated recombinant DNA was recovered.

(4) Genetic transformation with recombinant plasmid.

A histidine requiring strain, No. 200 (NRRL B-15047) which was derived from Corynebacterium glutamicum AJ 11560 by N-methyl-N'-nitro-N-nitrosoguanidine mutagenesis, (250 µg/ml in a 1/10 M phosphate buffer, pH 6.0 at 30°C for 60 minutes, and isolated as the histidine requiring mutant) was cultured in 20 ml of CMG medium at 30°C with shaking. Cells in exponential growth phase were harvested, and "competent" cells having the ability of DNA uptake were prepared by calcium chloride method. The calcium chloride method was conformed to that disclosed in Mandel, M and Higa,A., J. Mol. Biol., 53, 159 (1970). That is, No. 200 was inoculated into 20 ml CMG medium and cultured until the cell density reached 0.6 A650/ml. Cells were harvested, suspended in ice-cooled 0.1M $MgCl_2$, collected, suspended in 5 ml of 0.1 M $CaCl_2$ with ice-cooling, and held at 0°C for

20 minutes with shaking occasionally. The cells were separeted from the suspension suspended again in a small amount of 0.1M $CaCl_2$ and then obtained the competent cells suspension. Into the competent cell suspension, a solution of DNA obtained in step(3) was added to introduce the DNA into the cell. The reaction mixture was spread on the plate of M-medium supplmented with 0.1g/l L-histidine.

Colonies appeared on the plate after incubation at 37°C for 4 days and transformed to become AHV-resistant were picked up and L-threonine--producine transformants were selected.

Thus, AJ 11682 (FERM-P 5973= FERM BP 118) was obtained as the best threonine producing transformant.

(5) Production of L-threonine

L-Threonine productivity of AJ 11682 obtained in step (4) was tested comparing with the DNA-donor and DNA-recipient. The results are shown in Table 1.

The fermentation medium contained 10g/dl glucose, 3g/dl ammonium sulfate, 0.1g KH_2PO_4, 0.04g/dl $MgSO_4 \cdot 7H_2O$, 2mg/dl soyprotein hydrolysate ("MIEKI"), 10 µg/dl thiamine·HCl, 50µg/dl biotin, 1mg/dl $FeSO_4 \cdot 7H_2O$, 1mg/dl $MnSO_4 \cdot 4H_2O$ 10mg/dl L-histidine and 5g/dl $CaCO_3$ (separately sterilized) and the pH was adjusted to 7.2.

Twenty ml batches of the fermentation medium were placed in 500 ml flasks, inoculated with one loopful inoculum of the test microorganism, and the cultivation was carried out at 37°C for 72 hours.

The amount of L-threonine in the supernatant of the fermentation broth was determined by microbiological assay.

Table 1

Microorganism tested	L-threonine produced (mg/dl)
No. 60	140
No. 200	0
AJ 11682	295

Example 2

(1) Extraction of DNA

In the method shown in step(1) of Example 1, 2.1 mg of chromosomal DNA was obtained from a AHV resistant mutant, No.107 (NRRL B-15048) which had been derived from Brevibacterium lactofermentum ATCC 13869.

(2) Preparation of vector DNA

In the method shown in step(2) of Example 1 , 132 µg of a plasmid pAM 330 was separated from Brevibacterium lactofermentum ATCC 13869 as the vector DNA.

(3) Insertion of chromosomal DNA fragment into the vector

Ten µg of chromosomal DNA obtained in step(3) was digested by the manner shown in step(3) of Example 1. The vector DNA was also cutted by the manner shown in step(3), and the digested chromosomal DNA and the cutted vector DNA were subjected to the ligation reaction shown in step(3) of Example 1.

(4) Genetic transformation with the recombinant plasmid

From Brevibacterium lactofermentum ATCC 13869. No. S-18 (NRRL B-15049) which is resistant to streptomycine and requires

L-histidine was induced as the DNA-recipient by the method shown in step(4) of Example 1, a transformant, AJ 11683 (FERM-P 5974=FERM-BP 119) resistant to AHV and capable of producing L-threonine was obtained using the DNA-recipient.

(5) Production of L-threonine

The transformant AJ 11683 obtained in step(4) was tested its productivity of L-threonine by the method in step(5) of Example 1. The results are shown in Table 2.

Table 2

Microorganism tested	L-threonine produced (mg/dl)
No. 107	51
No. S-18	0
AJ 11683	132

PATENT CLAIMS

1. A method for producing L-threonine by fermentation which comprises aerobically culturing in an aqueous culture medium an L-threonine producing microorganism derived from a parent strain of the genus Brevibacterium or Corynebacterium and recovering L-threonine accumulated in the resulted culture liquid, characterized in that a strain transformed to become resistant to α-amino-ß-hydroxyvaleric acid is used, which is obtained by incorporation into a recipient strain of the genus Brevibacterium or Corynebacterium which is sensitive to α-amino-ß-hydroxy valeric acid, of a plasmid DNA obtained from a microorganism of the genus Brevibacterium or Corynebacterium and having been inserted therein a fragment of chromosomal DNA derived from a DNA-donor strain of the genus Brevibacterium or Corynebacterium resistant to α-amino-ß-hydroxyvaleric acid.

2. The method of claim 1, wherein said recipient strain belongs to Brevibacterium lactofermentum or Corynebacterium glutamicum.

3. The method of claim 1 or 2, wherein said DNA-donor strain belongs to Brevibacterium lactofermentum or Corynebacterium glutamicum.

4. The method of any of claims 1 to 3, wherein said DNA-donor strain is resistant to α-amino-ß-hydroxyvaleric acid in an amount of more than 100 µg/ml.

5. The method of any of claims 1 to 4, wherein said L-threonine producing microorganism is Corynebacterium glutamicum AJ 11682 (FERM-P 5973 = FERM BP 118) or Brevibacterium lactofermentum AJ 11683 (FERM-P 5974 = FERM BP 119).

6. A method for constructing an L-threonine producing strain which comprises:

(a) separating a plasmid DNA from a microorganism of the genus Brevibacterium and Corynebacterium,

(b) inserting into the plasmid DNA a fragment of chromosomal DNA derived from a DNA-donor strain of the genus Brevibacterium or Corynebacterium resistant to α-amino- - β-hydroxy valeric acid to obtain a recombinant plasmid DNA

(c) incorporating the recombinant plasmid DNA into a recipient strain of the genus Brevibacterium or Corynebacterium which is sensitive to α-amino- β-hydroxy valeric acid, and

(d) isolating a strain transformed to become resistant to α-amino- β-hydroxy-valeric acid.

7. The method of Claim 6, wherein said recipient strain belongs to Brevibacterium lactofermentum or Corynebacterium glutamicum

8. The method of claim 6 or 7, wherein said DNA-donor strain belongs to Brevibacterium lactofermentum or Corynebacterium glutamicum.

9. The method of claim 6, wherein said DNA-donor strain is resistant to an amount of L-threonine of more than 1000 µg/ml.

10. The method of claim 6, wherein said L-threonine producing microorganism is Corynebacterium glutamicum AJ 11682 (FERM-P 5973 = FERM-BP 118) or Brevibacterium lactofermentum AJ 11683 (FERM-P-5974 = FERM-BP 119).